

An integrative transcriptomic analysis reveals p53 regulated miRNA, mRNA, and lncRNA networks in nasopharyngeal carcinoma

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Abstract It has been reported that p53 dysfunction is closely related to the carcinogenesis of nasopharyngeal carcinoma (NPC). Recently, an increasing body of evidence has indicated that microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) participate in p53-associated signaling pathways and, in addition to mRNAs, form a complex regulation network to promote tumor occurrence and progression. The aim of this study was to elucidate the p53-regulated miRNAs, mRNAs, and lncRNAs and their regulating networks in NPC. Firstly, we overexpressed p53 in the NPC cell line HNE2 and performed transcriptomic gene expression profiling (GEP) analysis, which included miRNAs, mRNAs, and lncRNAs, using microarray technology at 0, 12, 24, and 48 h after transfection. There were 38 miRNAs (33 upregulated and 5 downregulated), 2107 mRNAs (296 upregulated and 1811 downregulated), and 1190 lncRNAs (133 upregulated and 1057 downregulated) that were significantly dysregulated by p53. Some of the dysregulated molecules were confirmed by quantitative real-time polymerase chain reaction (qRT-

PCR). Then, we integrated previously published miRNAs, mRNAs, and lncRNAs GEP datasets from NPC biopsies to investigate the expression of these p53 regulated molecules and found that 7 miRNAs, 218 mRNAs, and 101 lncRNAs regulated by p53 were also differentially expressed in NPC tissues. Finally, p53-regulated miRNA, mRNA, and lncRNA networks were constructed using bioinformatics methods. These miRNAs, mRNAs, and lncRNAs may participate in p53 downstream signaling pathways and play important roles in the carcinogenesis of NPC. Thorough investigations of their biological functions and regulating relationships will provide a novel view of the p53 signaling pathway, and the restoration of p53 functioning or its downstream gene regulating network is potentially of great value in treating NPC patients.

Keywords p53 · Nasopharyngeal carcinoma (NPC) · microRNAs (miRNAs) · mRNAs · Long noncoding RNAs (lncRNAs) · Gene regulating network

Zhaojian Gong and Qian Yang contributed equally to this work.

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Introduction

Nasopharyngeal carcinoma (NPC) is a common malignant tumor of the head and neck and particularly occurs in southern China and southeast Asian countries, with an annual incidence of 3–30 per 100,000 [1–3]. Due to its special anatomical position, radiotherapy has been the main clinical treatment [4]. The carcinogenesis of NPC is a complex process involving many genes, especially the activation of oncogenes and the inactivation of tumor suppressor genes [5–13].

TP53 gene (coding p53 protein) is an important tumor suppressor. It participates in the occurrence and development of a variety of malignant tumors through the regulation of a series of signal transduction pathways [14]. It is estimated that more than 50 % of tumors have *TP53* gene mutations, and that most tumors have abnormal p53 signaling pathways [15, 16]. p53 dysfunction is also closely related to the initiation and development of NPC and is significantly correlated with sensitivity to radiotherapy [17]. Therefore, *TP53* gene has been an

important candidate target for gene therapy, and restoring p53 function or its downstream signaling pathways will be of great significance to the treatment of NPC.

MicroRNAs (miRNAs) are a class of small endogenous noncoding RNAs (ncRNAs) that are 19–25 nucleotides in length and that, through specific binding of the mRNAs sequence of the 3'-untranslated region (UTR), induce the degradation [18–20] or inhibition of mRNA translation [21–26]. While long noncoding RNAs (lncRNAs) are a class of ncRNAs with molecular lengths of more than 200 nucleotides, the regulation of epigenetic gene expression occurs at transcriptional and post transcriptional levels and is involved in the regulation of various physiological and pathological processes [27–34]. MiRNAs and lncRNAs are involved in the carcinogenesis of multiple types of human cancers, and the abnormal expression or function disorder of miRNAs and lncRNAs are found in many tumors [35–41]. Some miRNA and lncRNA molecules have important significance in the diagnosis and treatment of tumors and can be used as new

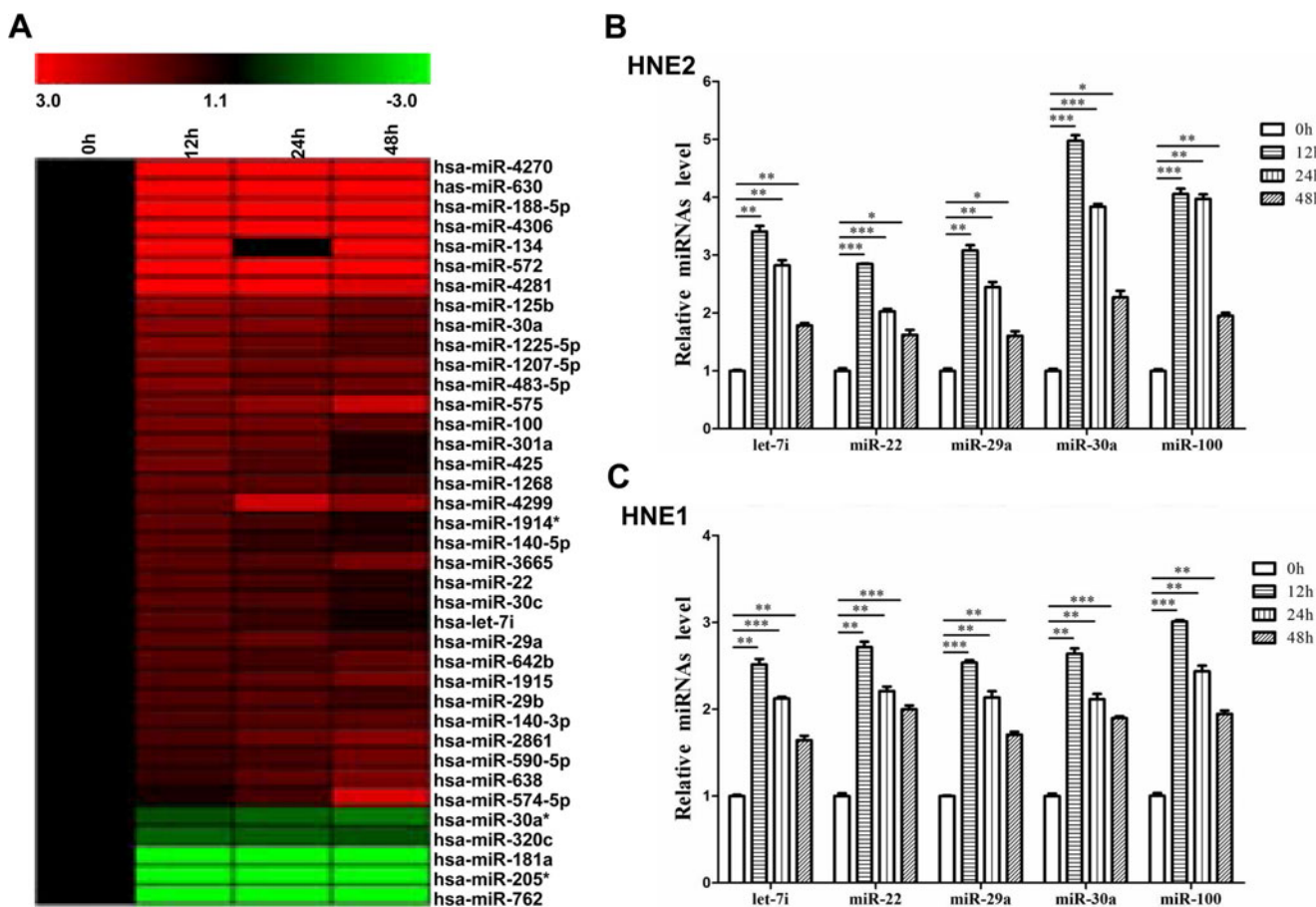
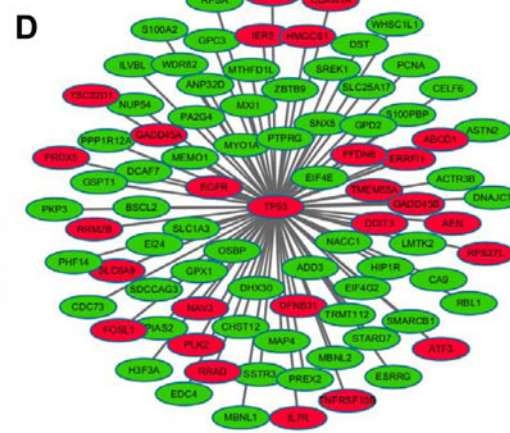
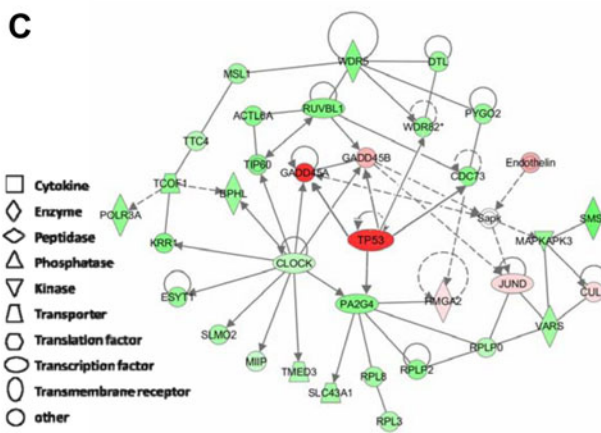
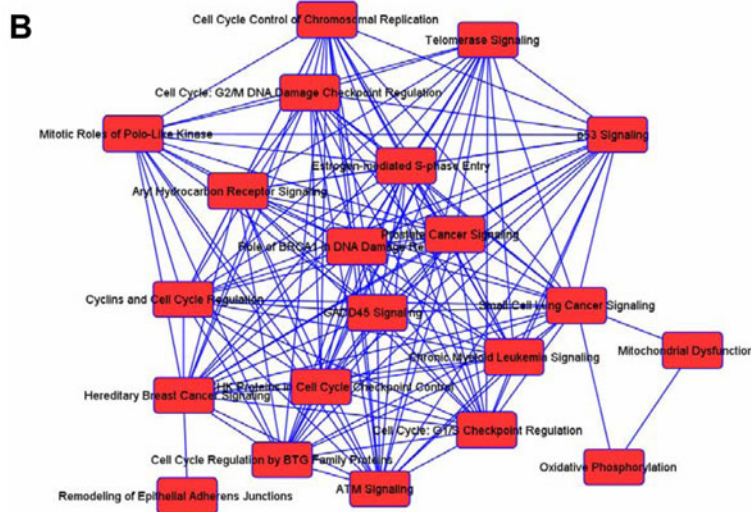
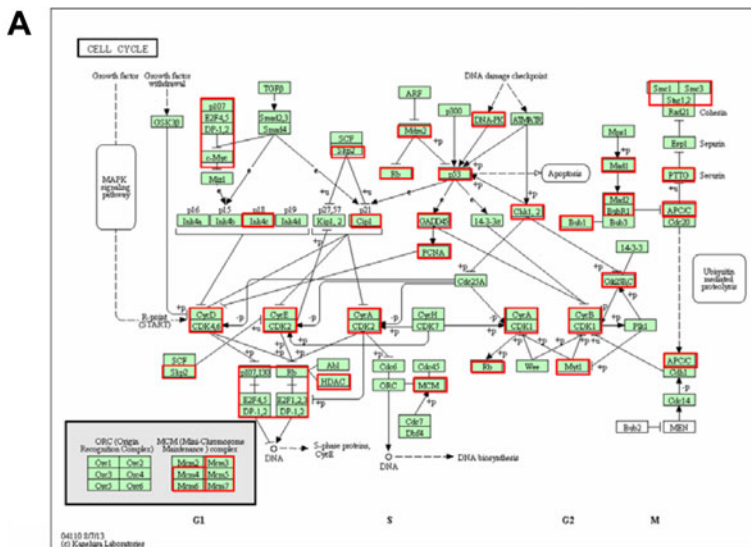


Fig. 1 Identification and confirmation of differentially expressed miRNAs regulated by p53 in HNE2 cells. **a** The differentially expressed miRNAs profiles in NPC HNE2 cells at 0, 12, 24, and 48 h after the p53 expression vector pCMV-p53 transfection. Red represents upregulation while green represents downregulation. The expression of miRNAs let-7i, miR-22, miR-29a, miR-30a, and miR-100 was confirmed

in **b** HNE2 and validated in another **c** NPC cell line HNE1. The expression levels of miRNAs at 0 h after the p53 expression vector pCMV-p53 transfection were used as the control and were normalized. The data was shown as the means of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



molecular markers for tumor prognosis [42–44]. An increasing number of events indicate that not only protein-coding genes [45] but also noncoding RNAs, including miRNAs [46–51] and lncRNAs, participate in p53-related gene-regulating networks and the carcinogenesis of a wide range of

cancers [52]. However, p53 downstream molecules, especially miRNAs and lncRNAs and their regulatory networks, have not been thoroughly investigated in NPC.

To build the miRNA, mRNA, and lncRNA interaction networks that are regulated by p53 in NPC, we first overexpressed

Fig. 2 The signaling pathways and networks of protein-coding genes (mRNAs) regulated by p53 in HNE2 cells. **a** p53-regulated protein-coding genes (mRNAs) were mainly involved in signaling pathways, such as the “cell cycle,” “p53 signaling,” “ATM signaling,” and “Gadd45 signaling.” The cell cycle signaling pathways are shown as a representation. Genes marked by a red rectangle were regulated and significantly differentially expressed in HNE2 cells after the p53 expression vector pCMV-p53 transfection. **b** p53-regulated signaling pathways have complex crosstalk and constitute larger networks. Each red node represents a signal pathway. **c** A network of p53-regulated mRNAs in the NPC cell line HNE2. The Ingenuity Pathway Analysis (IPA) software was used to analyze the p53 target genes. Four networks were identified. The network representing proteins involved in the “cell cycle, posttranslational modification, auditory, and vestibular system development and function” is shown. The intensity of a node color indicates the degree of its upregulation (*red*) and downregulation (*green*). The shape notes of each node are shown in the figure. **d** The network of *TP53* direct targeting genes mined from the GSEA database. The color of a node indicates upregulation (*red*) and downregulation (*green*)

TP53 gene in the NPC cell line HNE2 and then screened differentially expressed miRNAs, mRNAs, and lncRNAs using microarray technology. Through bioinformatics analyses, we found that these miRNAs, mRNAs, and lncRNAs that were regulated by p53 formed complex regulation networks and participated in the cell cycle, apoptosis, and other life activities. Combined with differentially expressed miRNA [53], mRNA [54], and lncRNA [55] gene expression profiling (GEP) datasets from NPC biopsies obtained from previously published papers, we found that some miRNAs and their target genes were regulated by *TP53* gene and were also differentially expressed in NPC, which indicated that they might play an important role in the occurrence and development of NPC through p53-related signaling pathways.

Materials and methods

Cell culture, transfection, and plasmids

Low-passage human NPC cell lines, HNE1 and HNE2, were maintained in RPMI-1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen) at 37 °C in a humidified 5 % CO₂ incubator. The cells were transfected with pCMV-p53 plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggested protocol. Cell cultures were grown under normal cell culture conditions for 12, 24, and 48 h and then harvested for total RNA extraction. The p53 expression vector pCMV-p53 was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). The luciferase plasmid, pp53-TA-luc, which was used for measuring p53 transcriptional activity, was purchased from the Beyotime Institute of Biotechnology (Beyotime, Shanghai, China). The pGL3-Enhancer plasmid and Renilla

luciferase plasmid pRL-TK vector used for the control were purchased from Promega (Madison, WI, USA).

Luciferase assay

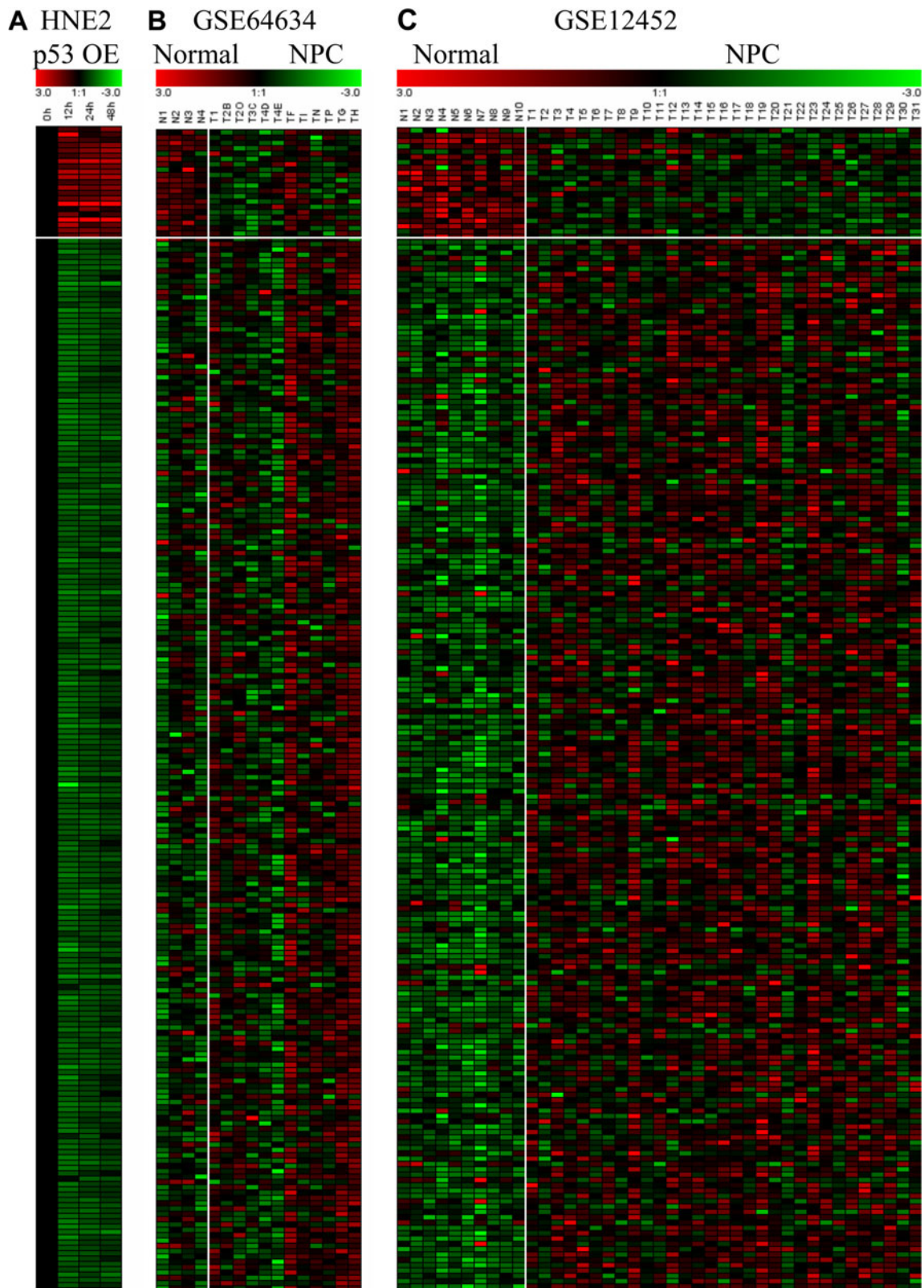
Luciferase assays were performed using the Dual-Glo Luciferase Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, cells were first co-transfected with pCMV-p53 and pp53-TA-luc plasmids, a luciferase reporter plasmid containing p53 binding sites used for assaying p53 transcriptional activity, in 24-well plates. After 0, 12, 24, or 48 h of transfection, cells were harvested and lysed in a buffer containing luciferase substrate. Renilla luciferase was used for normalization purposes. Transfection was performed in triplicates, and all experiments were repeated three times.

MiRNA, mRNA, and lncRNA expression profiling

The expression profiles were performed using an Agilent Human miRNA V16.0 and OE Biotech Human mRNA and lncRNA Microarray v2.0 [17]. Briefly, total RNA isolated from HNE2 cells at 0, 12, 24, or 48 h post transfection with pCMV-p53 were reverse transcribed using random hexamers and oligo-dT primers. Labeled cRNA was then synthesized and hybridized to a microarray. After hybridization and washing, the processed slides were scanned with an Agilent Microarray Scanner (Agilent Technologies, Santa Clara, CA, USA) and analyzed by Agilent Feature Extraction Software (Agilent Technologies). Quantile normalization and subsequent data processing were performed using the GeneSpring GX v. 11.0 software package (Agilent Technologies). The lncRNA and miRNA expression data were deposited in the Gene Expression Omnibus database (GEO, www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE60379 and GSE64629, respectively. Threshold fold changes >2 were used to screen for dysregulated miRNA, mRNA, and lncRNA.

RNA preparation and qRT-PCR

Total RNA was extracted using the TRIzol extraction Kit (Invitrogen). For miRNA expression, a SYBR PrimeScript™ miRNA RT-PCR kit (Takara, Dalian, China) was used to synthesize cDNA and qRT-PCR. The following primers were used: hsa-let-7i-5p, 5'-TGAGGTAGTAGTTTGTGCTGTT-3'; hsa-miR-22-3p, 5'-AAGCTGCCAGTTGAAGAACTGT-3'; hsa-miR-29a-3p, 5'-TAGCACCATCTGAAATCGGTTA-3'; hsa-miR-30a-5p, 5'-TGTAACATCCTCGACTGGAAG-3'; hsa-miR-100-5p, 5'-AACCCGTAGATCCGAACCTTGTG-3'; and U6, 5'-ATTGGAACGATACAGAGAAGATT-3' and 5'-GGAACGCTTACGAATTTG-3'. U6 was used to normalize the expression level of miRNAs. The expression of *TP53* and its target genes *CDKN1A* as well as *MDM2* were also



determined by qRT-PCR using the SYBR Green method with specific primers *TP53*, 5'-CCCTTCCCAGAAAACCTACC-3' and 5'-CTCCGTCATGTGCTGTGACT-3'; *CDKN1A*, 5'-AGGGGACAGCAGAGGAAGAC-3' and 5'-

GGCGTTTGGAGTGGTAGAAA-3'; and *MDM2*, 5'-GTATCAGGCAGGGGAGAGTG-3' and 5'-GAAGCCAATTCTCACGAAGG-3'. Primers targeting β -actin mRNA (5'-GCATCCCCCAAAGTTCACAA-3' and 5'-

Fig. 3 Integrated analyses of p53-regulated mRNAs both differentially expressed in HNE2 cells and NPC primary tumors. The expression of 296 upregulated and 1811 downregulated mRNAs that were identified in HNE2 cells after p53 transfection were investigated in two independent cohorts of NPC primary tumor biopsies that were previously published [27, 54]. We found 21 mRNAs that were upregulated by p53 and also downregulated in NPC tissues compared to normal tissues (upper panel), while 197 mRNAs were downregulated by p53 and also upregulated in NPC tissues. The heat map of their expression in HNE2 cells (a) and 2 NPC GEP datasets GSE64634 (b, constructed by our lab [27]) and GSE12452 (c, published previously [54]) are shown

AGGACTGGGCCATTCTCCTT-3') were used as internal controls for mRNAs.

The construction of p53 downstream miRNA, mRNA, and lncRNA regulatory networks

Ingenuity pathways analysis (IPA) software was used to identify signaling pathways regulated by p53 mining from our microarray data (GSE64634) [27] and the GEO database (GSE12452) [54]. p53 targeting genes were obtained from the gene set enrichment analysis (GSEA) database [10]. Dysregulated miRNAs in NPC were screened from our own miRNA chip [53]. MiRNA target genes were predicted by TargetScan, miRecords, TarBase, and miRecords software [56]. The putative miRNA binding sites of the lncRNAs sequences were performed using the RegRNA software ([\[regrna2.mbc.nctu.edu.tw/\]\(http://regrna2.mbc.nctu.edu.tw/\)\) \[57\]. The p53 downstream miRNA-mRNA-lncRNA interaction network was built with the Cytoscape Software \(version 3.1.0\) \[58\].](http://</p>
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Statistical analysis

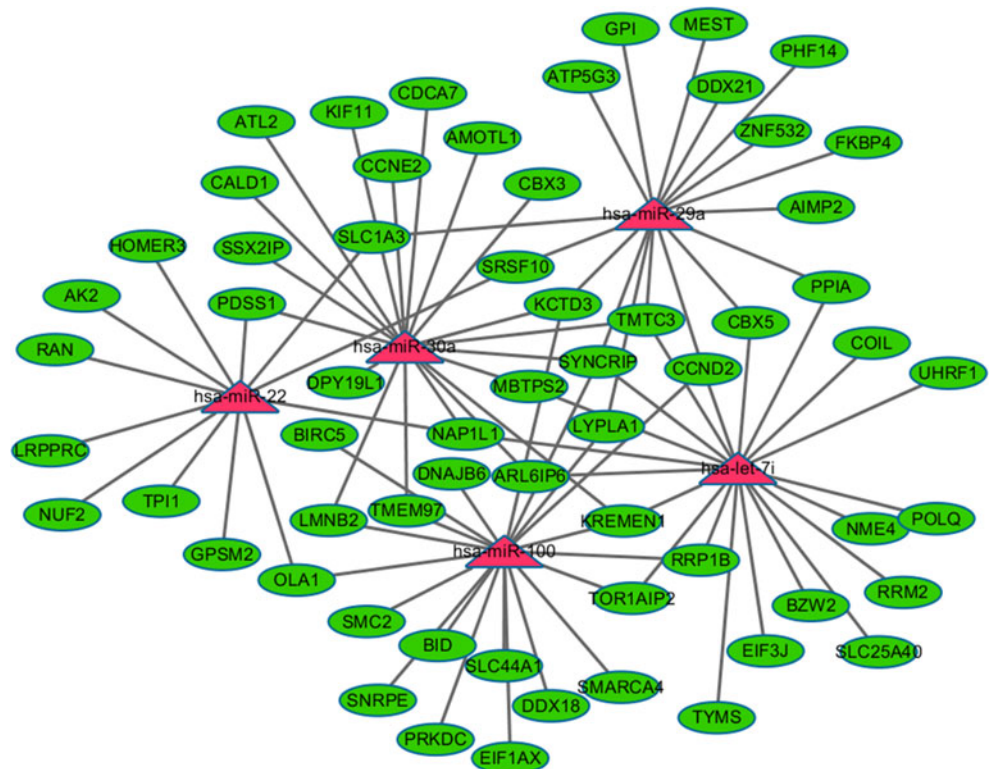
Significant differences in the gene expression levels, cell survival rates, and proliferation rates between groups were analyzed by ANOVA, and the results are expressed as the mean \pm standard deviations (SD). *P* values <0.05 were considered statistically significant, and all statistical tests were two-sided.

Results

Ectopic expression of p53 in the NPC cell line HNE2

The NPC cell line HNE2 was transiently transfected with the p53 expression vector pCMV-*TP53* and collected 0, 12, 24, and 48 h after transfection. Then, we confirmed p53 expression and its transcriptional activity firstly. qRT-PCR and western blotting showed that the expression of p53 was significantly induced after transfection at both the mRNA and protein levels. A luciferase assay also confirmed that the p53 transcriptional activity was increased significantly after the p53 expression vector pCMV-*TP53* transfection (Supplemental Fig. S1).

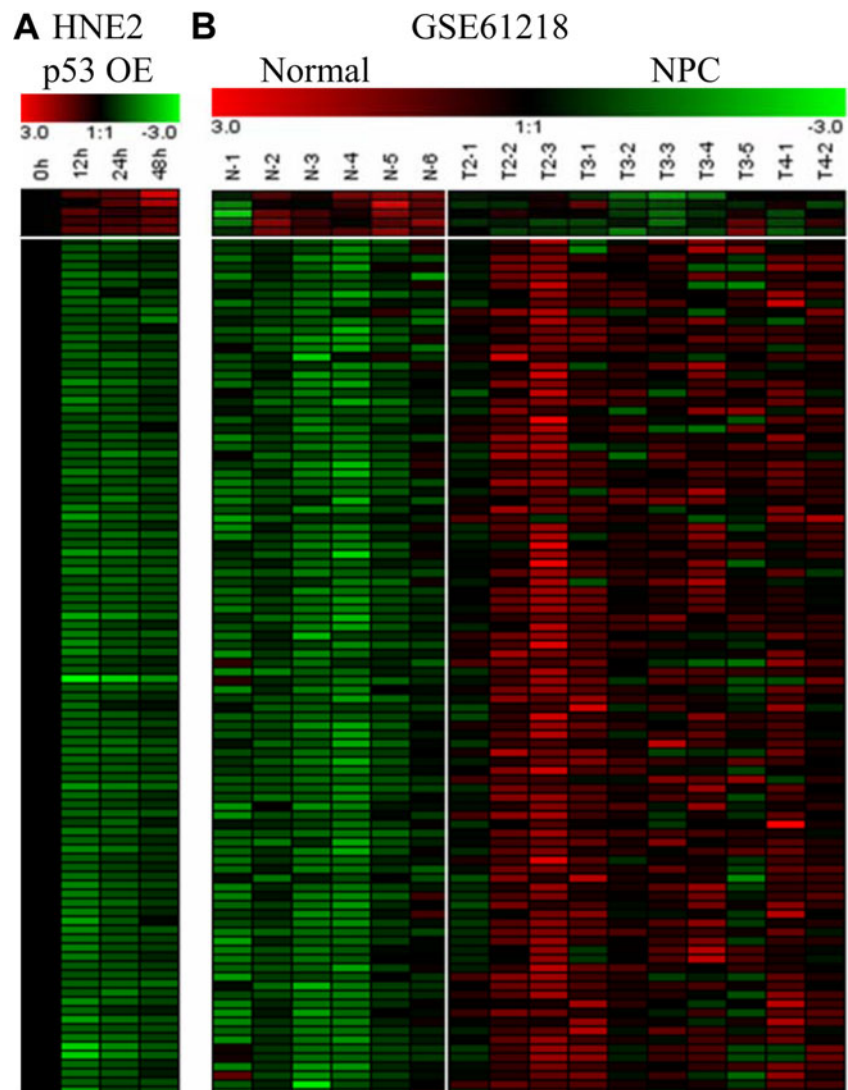
Fig. 4 The miRNA networks and their targeted mRNAs regulated by p53 in NPC. The expression levels of miRNAs and mRNAs presented were analyzed in NPC biopsies. Only those p53-regulated miRNAs and mRNAs with a significantly differential expression pattern in NPC biopsies were left out. Next, a small interaction network between has-let-7i, has-miR-100, has-miR-22, has-miR-29a, and has-miR-30a and their target genes was constructed by the Cytoscape software. The circle node represents mRNA, and the triangle node represents miRNA; red represents upregulation while green represents downregulation



The miRNAs expression profiling regulated by p53

The miRNA expression profiling was performed in HNE2 cells after 0, 12, 24, and 48 h of transfection (GSE64629). There were 33 miRNAs significantly upregulated and 5 miRNAs downregulated by p53 (Fig. 1a, Supplemental Table S1). Among these differentially expressed miRNAs, hsa-let-7i, hsa-miR-100, hsa-miR-22, hsa-miR-29a, hsa-miR-29b, and hsa-miR-30a were downregulated in NPC biopsies, while miR181a was upregulated compared to normal nasopharyngeal epithelium (NPE) in previously published studies [59–62]. In particular, it was reported that hsa-miR-22 [63] and hsa-let-7i [64] are direct targets of p53. We then confirmed the expression of hsa-let-7i, hsa-miR-100, hsa-miR-22, hsa-miR-29a, and hsa-miR-30a in the NPC cell line HNE2 (Fig. 1b) and their expression in another NPC cell line HNE1 (Fig. 1c) by qRT-PCR. The findings were all consistent with our microarray data.

Fig. 5 Integrated analyses of p53 regulated lncRNA both differentially expressed in HNE2 cells and NPC primary tumors. The expression of 133 upregulated and 1057 downregulated lncRNAs identified in HNE2 cells after p53 transfection were investigated in the GEP data of NPC primary tumor biopsies. Finally, we found 5 lncRNAs that were upregulated by p53 and also downregulated in NPC tissues compared to normal tissues (upper panel), while 96 mRNAs were downregulated by p53 and also upregulated in NPC tissues. The heat map of their expression in HNE2 cells (a) and NPC GEP datasets GSE61218 (b, constructed by our lab) is presented here



The mRNA expression profile regulated by p53

The mRNA expression profiling was also performed in HNE2 cells at 0, 12, 24, and 48 h after p53 transfection. A total of 296 protein-coding genes (mRNAs) were significantly induced, and 1811 mRNAs were reduced by p53 (Supplemental Table S2). These genes were mainly involved in signaling pathways, such as the “cell cycle” (Fig. 2a), “p53 signaling,” “ATM signaling,” and “Gadd45 signaling.” Additionally, these signaling pathways also had complex crosstalk (Fig. 2b) when we analyzed and annotated the dysregulated genes with the IPA software. Moreover, constructed by IPA, these differentially expressed genes formed regulatory networks that were involved in the regulation of the cell cycle progression and apoptosis (Fig. 2c and Supplemental Figure S2).

Integrated with *TP53* target gene sets from the GSEA database [10], we found that there were 21 upregulated

genes, such as *CDKN1A* (encoding p21), *MDM2*, *GADD45A*, and *GADD45B*, and 40 downregulated genes (such as *PCNA*, *EIF4E*, *SSTR3*, and *ANP32D*) with p53 binding sites in their promoter region, and their transcription may be directly regulated by p53 (Fig. 2d). The expression levels of *CDKN1A* and *MDM2* were validated by real-time PCR (Supplemental Figure S3) and were consistent with our microarray data.

Next, we investigated whether these p53 regulated mRNAs were abnormally expressed in NPC biopsies. We analyzed the

gene expression profiles of two independent NPC cohorts: GSE64634 was constructed by our lab [27] and GSE12452 was published previously [54] and downloaded from the GEO database. Twenty-one mRNAs, including *ADSSLI*, *AKR1C3*, *ALDH3A1*, and *CFB*, were upregulated by p53 (Fig. 3a, upper panel) and also downregulated in NPC tissues compared to normal tissues (Fig. 3b, c, upper panel); 197 mRNAs, including *CCND2*, *CDK1*, *CDK4*, *CFLAR*, and *MYC* were downregulated by p53 (Fig. 3a, lower panel) and also upregulated in NPC tissues (Fig. 3b, c, lower panel), which suggests that these

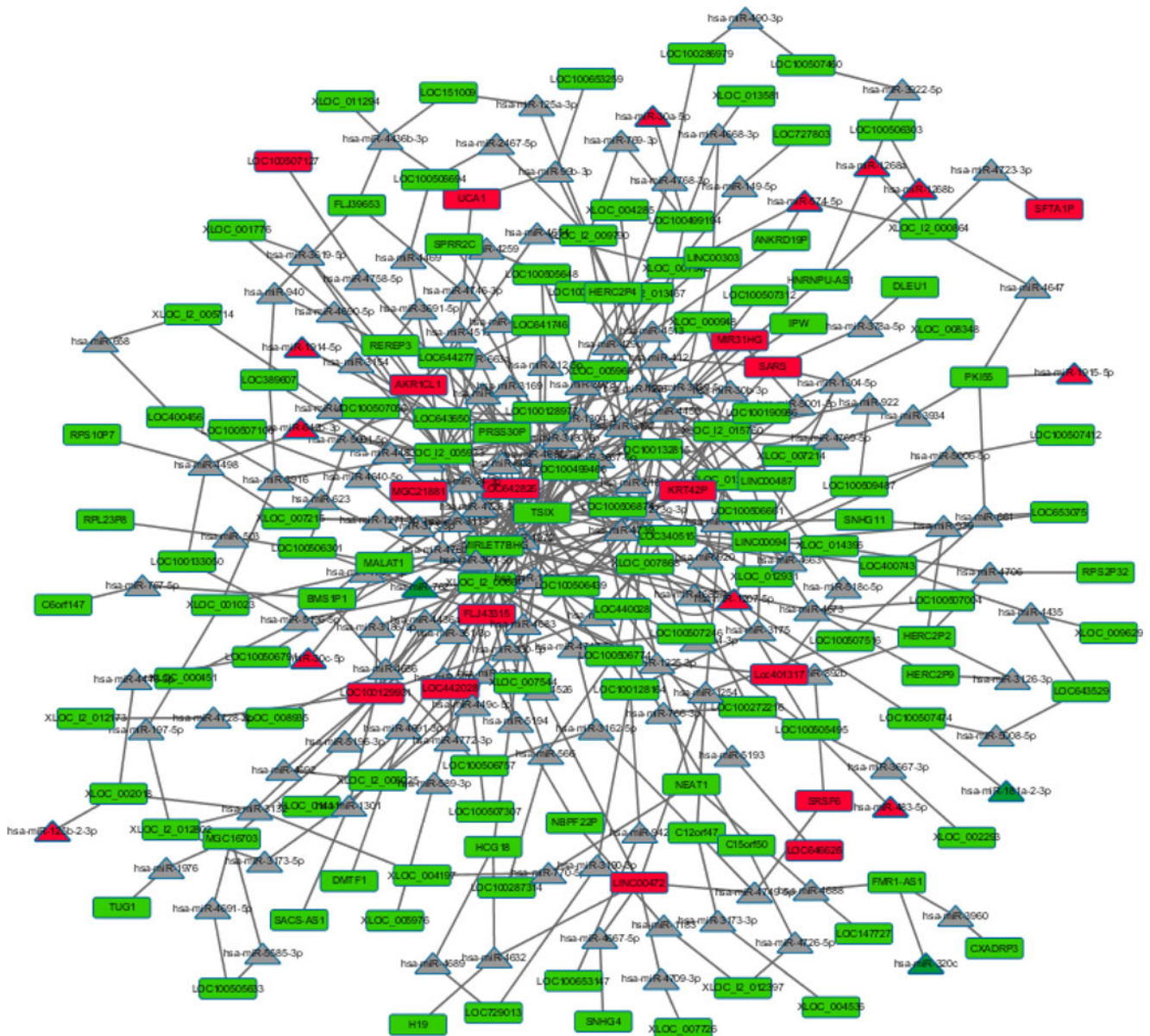


Fig. 6 The interaction network between lncRNAs and their target miRNAs predicted by the RegRNA software. To construct miRNA-lncRNA regulating networks, we predicted the miRNA binding sites on the 101 differentially expressed lncRNAs shown in Fig. 5 using the RegRNA 2.0 software. We identified 908 miRNA-lncRNA pairs, and these 908 miRNA-lncRNA pairs were constructed into a miRNAs-

lncRNAs network with the Cytoscape software. The round rectangle node represents the lncRNA, and the triangle node represents the miRNA; the red node represents upregulation, green represents downregulation, and grey indicates that the differential expression did not reach statistical significance

genes might be regulated by p53 and participate in the carcinogenesis of NPC.

The miRNA-mRNA networks regulated by p53

To construct the interaction network between miRNAs and mRNAs regulated by p53 in HNE2 cells, we further analyzed the mutual regulation of differentially expressed miRNAs and mRNAs. Because miRNAs can induce the degradation of their target mRNAs, we focused on those miRNAs and mRNAs with contrasting expression patterns. There were 33 upregulated miRNAs and 1911 downregulated mRNAs, as well as 5 downregulated miRNAs and 296 upregulated mRNAs, in p53-transfected HNE2 cells that were entered into the IPA software, and 2534 potential miRNAs-mRNAs pairs were identified among these opposite differentially expressed miRNAs and mRNAs using the miRNA target screening strategy. Most of these miRNAs targeting mRNAs also participate in the cell cycle and DNA damage repair, and a p53-regulated

miRNA-mRNA network was constructed by a cystoscape (Supplemental Figure S4).

We then analyzed the expression patterns of these 2534 miRNAs-mRNAs pairs in NPC biopsies based on previously published datasets [53, 54]. There were five miRNAs (hsa-let-7i, hsa-miR-22, hsa-miR-29a, hsa-miR-30, and hsa-miR-100) induced by p53 that were downregulated in NPC tissues, and their 115 target mRNAs were also inhibited by p53 in HNE2 cells but upregulated in NPC tissues (Fig. 4), suggesting that the potential regulation network between these miRNAs and mRNAs may play more important role in the carcinogenesis of NPC.

p53 downstream miRNA, mRNA, and lncRNA regulatory networks in HNE2 cells

LncRNA expression profiling was also performed in HNE2 cells at 0, 12, 24, and 48 h after p53 transfection. A total of 133 lncRNAs were upregulated, and 1057 lncRNAs were

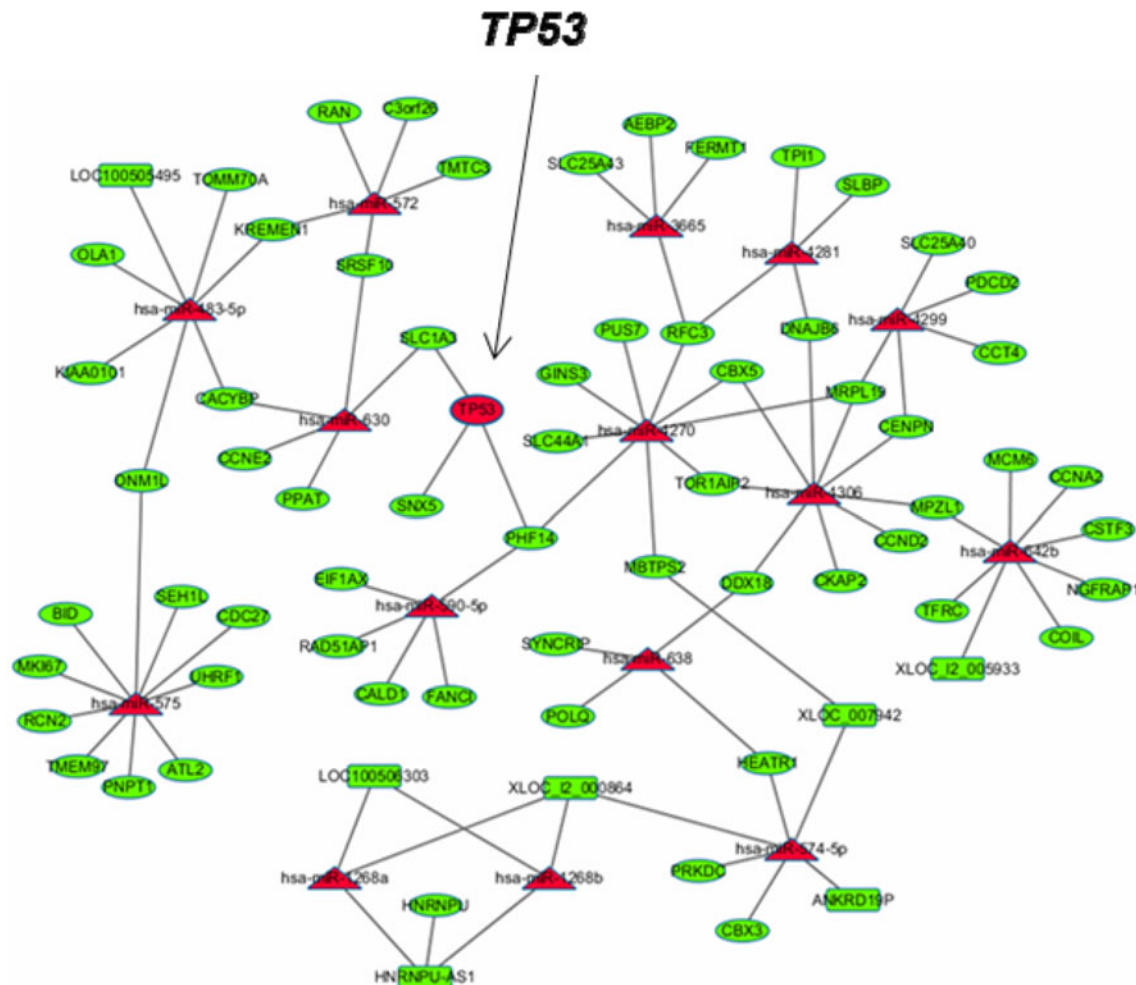


Fig. 7 The miRNA, mRNA, and lncRNA interaction networks regulated by TP53 in HNE2 cells. The circle nodes represent the mRNA, the triangle nodes represent the miRNA, the round rectangle nodes

represent the lncRNA, and the red nodes represent upregulation while the green nodes represent downregulation

downregulated by p53 (Supplemental Table S3). We investigated whether these p53-regulated lncRNAs were abnormally expressed in NPC biopsies. Compared to our lncRNA expression profiles from NPC biopsies (GSE61218), there were 5 lncRNAs induced by p53 in HNE2 cells and downregulated in NPC biopsies, which may act as tumor suppressors in NPC carcinogenesis, while 96 lncRNAs were inhibited by p53 and upregulated in NPC tissues, which may act as oncogenes (Fig. 5).

To construct the miRNA-lncRNA regulating networks, we predicted the miRNA binding sites on these 101 differentially expressed lncRNAs using the RegRNA 2.0 software. We identified 908 miRNA-lncRNA pairs, and most lncRNAs were targeted by the same miRNA with other lncRNAs. These 908 miRNAs-lncRNAs pairs were constructed into a miRNA-lncRNA network (Fig. 6). As most of the miRNAs that could target lncRNAs in Fig. 6 were not differentially expressed in HNE2 cells after p53 overexpression, we focused on the miRNA-lncRNA pairs that were both regulated by p53 in HNE2 cells and dysregulated in NPC biopsies and combined them with the miRNA-mRNA network illustrated in Fig. 4b. Finally, we constructed a p53 regulated miRNA-mRNA-lncRNA interaction network (Fig. 7). This miRNA-mRNA-lncRNA network was not only a target of p53 in the NPC cell line HNE2, but was also significantly differentially expressed in NPC biopsies.

Discussion

p53 dysfunction is critically evident in the carcinogenesis of NPC. It is closely related to the NPC tissue proliferation degree, the density of blood vessels, and the invasion of the skull base, neck lymph node metastasis, and patient prognoses. Therefore, p53 is a very important target for NPC therapy. If p53 or its downstream signaling pathways can be restored, it will be of great relevance to the treatment of NPC patients [65].

TP53 gene is a very strong tumor suppressor gene, and overexpressed p53 can rapidly induce apoptosis in NPC cells. In this study, we firstly overexpressed p53 in HNE2 cells through the transiently transfected p53 expression vector pCMV-p53. The level of p53 protein reached its peak at 24 h after transfection, while expression of p53 target genes such as *CDKN1* and *MDM2* reached their peaks at 12 h and earlier than the p53. We think this may be because NPC cells had already started apoptosis at 12 h after transfection. We identified p53 regulated miRNAs, mRNAs, and lncRNAs through the ectopic expression of *TP53* gene in NPC cells. Through integrative miRNA, mRNA, and lncRNA expression profiling, we found that several miRNAs (hsa-let-7i, hsa-miR-22, hsa-miR-29a, hsa-miR-29b, hsa-miR-30a and hsa-miR-100) were upregulated by p53 and were also downregulated in NPC biopsies, indicating that these miRNAs might be

acting as tumor suppressors in occurrence or progression of NPC. In contrast, miR181a was downregulated by p53 and upregulated in NPC biopsies, implying that it may act as an oncogene. Hsa-miR-22 is a direct target gene of p53 [63] and promotes tumorigenesis and metastasis through the inhibition of tumor suppressor genes *PTEN* and *TET* [66]. let-7i is also a transcriptional target of p53, and the introduction of let-7i to mutant p53 cells significantly inhibited migration, invasion, and metastasis by repressing a network of oncogenes, including *E2F5*, *LIN28B*, *MYC*, and *NRAS* [64]. Mechanism of p53-induced expressions of hsa-miR-29a, hsa-miR-30a and hsa-miR-100 have not been reported and is worth further study. We also constructed an interaction network including hsa-let-7i, hsa-miR-22, hsa-miR-29a, hsa-miR-30a, and hsa-miR-100 and their potential target mRNAs and lncRNAs. These miRNAs, mRNAs, and lncRNAs were not only regulated by p53 but were also abnormally expressed in NPC tissues, which means that they may play critical roles in the carcinogenesis of NPC through p53-related signaling pathways.

Regarding the p53-regulated mRNAs and lncRNAs, it is interesting that the number of p53-inhibited mRNAs (1811) and lncRNAs (1057) were much greater than the number of p53-induced mRNAs (296) and lncRNAs (133). The number of p53-induced miRNAs (33) was greater than the number of p53-inhibited miRNAs (only 5), which is reasonable because miRNAs can bind to and degrade their target mRNAs or lncRNAs; we hypothesize that most of those mRNAs and lncRNAs were targeted and regulated by miRNAs driven by p53.

For the p53-regulated mRNAs, there were 197 mRNAs, including *CCND2*, *CDK1*, *CDK4*, *CFLAR*, and *MYC* that were downregulated by p53 and also upregulated in NPC tissues. We hypothesize that these genes were mainly involved in signaling pathways, including “p53 signaling,” “cell cycle,” “ATM signaling,” and “Gadd45 signaling,” suggesting that the GEP results were reliable. These mRNAs such as *CCND2*, *CDK1*, *CDK4*, *CFLAR*, and *MYC* were the main molecules in the cell cycle pathway, DNA damage repair, cell apoptosis, and other signaling pathways. P53 induced the expression of these molecules, and then induced the cell cycle, DNA damage repair, cell apoptosis, and other signaling pathways involved in p53. Abnormal proliferation and/or expression deletion of these molecules can make the cell cycle G1/S checkpoint and cell apoptosis out of control, resulting in unlimited cell carcinogenesis, such as NPC [67–69]. Using the IPA software, we also constructed novel p53-regulated protein-coding gene networks (Fig. 2c and Supplemental Figure S2), but the mechanisms through which p53 drives these networks and participates in the carcinogenesis of NPC remain unknown. Does p53 transcription directly regulate its downstream protein-coding genes or are the p53 transcript miRNAs regulated by downstream protein coding genes mediated by miRNAs? These questions require further investigation.

At present, more than 50,000 lncRNA genes have been identified [70], which is much greater than the number of protein-coding genes (approximately 21,000); however, only a very limited number of lncRNAs have been studied and have biological function annotations. Among the p53-regulated lncRNAs identified in this study, some lncRNAs, including *lincRNA-p21* [71], *PANDA* [72], *H19* [73, 74], *LOC285194* [75], and *LOC401317* [17], were already reported to be regulated by p53, although many more p53-regulated lncRNAs and their function in NPC occurrence and progression have yet to be elucidated. Professor Pandolfi presented the hypothesis of “competing endogenous RNA” (ceRNA), or crosstalk between RNAs, both coding and noncoding, through microRNA response elements (MREs) to form large-scale regulatory networks across the transcriptome, greatly expanding the functional genetic information of the human genome [76, 77]. For example, hsa-miR-30a and LOC100499194 interact with each other in these networks. LOC100499194 may combine with hsa-miR-30a through competition with other target mRNAs. In this study, most lncRNAs and mRNAs share the same miRNAs, and our study provided valuable clues for the identification of potential ceRNA pairs, between lncRNAs and lncRNAs, lncRNAs, and mRNAs, or even mRNAs and mRNAs.

To our knowledge, this is the first study to construct interaction networks among a series of miRNAs, mRNAs, and lncRNAs downstream of p53 in NPC. It will be helpful to further illustrate the biological function of p53 by thoroughly investigating its downstream miRNA, mRNA, and lncRNA networks. These miRNAs, mRNAs, and lncRNAs may play important roles in the carcinogenesis of NPC, and their potential regulatory relationships and detailed participation mechanisms in p53 signaling pathways and NPC carcinogenesis require further investigation.

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Compliance with ethical standards

Conflicts of interest None

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